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Tenth day of September 2010

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PROVISIONAL SPECIFICATION

Invention Title: "NEISSERIA GONNORRHOEAE
DETECTION"

The invention is described in the following statement:

TITLE

NEISSERIA GONNORRHOEAE DETECTION

FIELD OF THE INVENTION

THIS INVENTION relates to detection of the bacterium *Neisseria gonorrhoeae*, or
5 more particularly to detection of bacterial nucleic acids. This invention particularly
relates to the detection of amplified fragments of a *porA* gene of *Neisseria*
gonorrhoeae for determining whether the bacterium is present in a biological
sample obtained from an individual, typically for the purposes of clinical diagnosis.

BACKGROUND OF THE INVENTION

10 *Neisseria gonorrhoeae* is a gram-negative diplococcal bacterial pathogen
which is the causative organism of the sexually transmitted disease (STD) known as
"gonorrhoeae". Although *gonorrhoeae* is an ancient disease first described by
Galen in AD 150, it is still a major STD of humans. Failure to detect and treat
Neisseria gonorrhoeae infection can allow the disease to progress to a serious
15 systemic infection that affects the heart, joints, meninges, eyes and pharynx. Thus,
early definitive diagnosis can assist treatment of the disease and prevent the serious
complications that can arise as a result of this bacterial infection.

Although polymerase chain reaction (PCR) is the method of choice for
routine detection of *Neisseria gonorrhoeae*, PCR has limitations and bacterial
20 isolation has remained the gold standard for definitive diagnosis. This is largely
because *N. gonorrhoeae* shares much sequence homology with other *Neisseria*
species, including *N. meningitidis*, and in addition, contains many non-conserved
sequences (Palmer et al 2003). Thus, there is a potential for both false-positive and

false-negative results to occur when using PCR for routine detection of *N. gonorrhoeae*. In both situations the consequences may be significant. From a public health perspective, false-negative results may allow unchecked spread of the disease whereas false-positive results can have considerable social ramifications for patients.

5 The Roche Cobas Amplicor system (Roche Diagnostics, Australia) is a PCR assay widely used for the detection of *N. gonorrhoeae*. Its appeal lies in its ability to simultaneously detect *N. gonorrhoeae*, *Chlamydia trachomatis* and the presence of inhibiting substances, while also carrying United States Food and Drug Administration (FDA) approval. However, the Cobas Amplicor system does have
10 limitations. Most notably, its *N. gonorrhoeae* assay is known to cross react with some strains of commensal Neisseria species, including *N. subflava*, *N. cinerea*, *N. flavescens*, *N. lactamica* and *N. sicca* (Palmer et al 2003; Farrell 1999). Consequently, there is a need to use a second PCR assay to confirm Cobas Amplicor positive results. In response, clinical laboratories have adopted in-house confirmatory
15 assays.

To date, the most common target for in-house confirmatory tests has been the cryptic plasmid (cppB) gene of *N. gonorrhoeae*, with several such protocols having been described (Ho et al 1992; Farrell 1999; Whiley et al 2002, Tabrizi et al 2004). In particular, a LightCycler based cppB PCR (cppB-LC) assay has been developed
20 for confirmation of Cobas *N. gonorrhoeae* positive specimens (Whiley et al 2002). However, serious concerns have now been raised over the sensitivity and specificity of *N. gonorrhoeae* assays targeting the cppB gene. Studies conducted in both the United Kingdom and Australia have identified *N. gonorrhoeae* isolates lacking the

cppB gene (Palmer et al 2003, Tapsall et el. 2003). Therefore, laboratories targeting this gene run the risk of false-negative results. In addition, Palmer et al.(2003) found that the cppB gene could be present in commensal *Neisseria* strains, including *N. cinerea*, and so could also produce false-positive results.

5

SUMMARY OF THE INVENTION

The present invention is therefore broadly directed to an method of determining whether an individual is or has been infected with *Neisseria gonorrhoeae*, which method utilizes a *porA* pseudogene or *porA* nucleic acid derived therefrom, as an indicator of infection.

10

The present invention is also broadly directed to one or more oligonucleotides which facilitate detection of a *Neisseria gonorrhoeae porA* gene or *porA* nucleic acid.

15

In a first aspect, the invention provides a method of determining whether an individual is or has been infected with *Neisseria gonorrhoeae*, said method including the step of detecting an isolated *porA* nucleic acid of *Neisseria gonorrhoeae* if present in a biological sample obtained from said individual, a presence of said *porA* nucleic acid indicating that said individual is or has been infected with *Neisseria gonorrhoeae*.

20

Preferably, the method includes the step of subjecting a nucleic acid sample to nucleic acid sequence amplification under conditions which facilitate amplification of said isolated *porA* nucleic acid to a detectable level.

In a second aspect the invention provides a method of determining whether an individual is or has been infected with *Neisseria gonorrhoeae*, said method

including the step of selectively detecting an isolated *porA* nucleic acid of *Neisseria gonorrhoeae* from a *porA* nucleic of another *Neisseria* species if present in said biological sample obtained from said individual, a presence of said isolated *porA* nucleic acid indicating that said individual is or has been infected with *Neisseria*
5 *gonorrhoeae*.

Preferably, the method includes the step of subjecting a nucleic acid sample to nucleic acid sequence amplification under conditions which facilitate amplification of said isolated *porA* nucleic acid to a detectable level but which do not facilitate amplification of said *porA* nucleic of said another *Neisseria* species to a
10 detectable level.

Preferably, said another *Neisseria* species is *N. meningitidis*.

In a preferred embodiment the invention provides a method of determining whether a human individual is or has been infected with *Neisseria gonorrhoeae*, said method including the steps of:

15 (i) subjecting a biological sample obtained from said human individual to nucleic acid sequence amplification to selectively produce a *porA* *Neisseria gonorrhoeae* amplification product from a *Neisseria gonorrhoeae porA* nucleic acid if present in said biological sample; and

(ii) detecting said amplification product, if present, by probe hybridization
20 whereby a presence of said *porA* amplification product indicates that said individual is or has been infected with *Neisseria gonorrhoeae*.

In a third aspect, the invention provides an oligonucleotide which is capable of hybridizing to a *porA* nucleic acid of *Neisseria gonorrhoeae* sufficiently to enable detection of said *porA* nucleic acid, but which is not capable of hybridizing to a *porA* nucleic acid of another *Neisseria* species sufficiently to enable detection of said *porA* nucleic acid of said another *Neisseria* species.

Preferably, said another *Neisseria* species is *N. meningitidis*.

In preferred embodiments, said oligonucleotide comprises a nucleotide sequence as set forth in Table 1 and FIG. 1.

In a fourth aspect, the invention provides a kit comprising one or more oligonucleotides according to the third aspect together with a DNA polymerase and/or one or more detection reagents.

In a fifth aspect the invention provides a nucleic acid array comprising an oligonucleotide according to the second aspect, immobilized, coupled, impregnated or otherwise in communication with a substrate.

It will be appreciated that the invention provides a method and oligonucleotide that facilitate detection of a *porA* nucleic acid of *Neisseria gonorrhoeae*.

Preferably the *porA* nucleic acid corresponds to a fragment of a *porA* pseudogene of *Neisseria gonorrhoeae*.

More preferably, according to this embodiment the *porA* nucleic acid corresponds to a fragment of a *porA* pseudogene of *Neisseria gonorrhoeae*, which fragment has a nucleotide sequence distinct from a fragment of a *porA* gene or pseudogene of another *Neisseria* species.

In a particularly preferred embodiment, the *porA* nucleic acid of *Neisseria gonorrhoeae* comprises a nucleotide sequence to which an oligonucleotide is capable of annealing sufficient to enable detection of said *porA* nucleic acid.

Suitably, said nucleotide sequence is not present in, or has one or more
 5 nucleotides different to, a nucleotide sequence of a *porA* nucleic acid of another *Neisseria* species, such that said oligonucleotide is not capable of hybridizing to said *porA* nucleic acid of said another *Neisseria* species sufficient to facilitate detection of said *porA* nucleic acid of said another *Neisseria* species.

Preferably, said another *Neisseria* species is *N. meningitidis*.

10 In a particularly advantageous embodiment, the method and oligonucleotide of the invention facilitate detection of a *porA* nucleic acid that may be present in each of a plurality of isolates, strains, allelic variants or sub-species of *Neisseria gonorrhoeae*.

Throughout this specification, unless otherwise indicated, "comprise",
 15 "comprises" and "comprising" are used inclusively rather than exclusively, so that a stated integer or group of integers may include one or more other non-stated integers or groups of integers.

BRIEF DESCRIPTION OF THE FIGURES

FIG.1 List of preferred oligonucleotide primer sequences (AS = antisense),
 20 and nucleotide sequence of *N. gonorrhoeae porA* pseudogene. Bolded residues indicate (5'to 3') G-pap-F (forward) primer annealing site; G-pap-p1 probe hybridization site; G-pap-p2 probe hybridization site; and G-pap-R (reverse) primer annealing site. The expected amplification product size is 132 bp. Shaded residues

are those which are non-identical with the corresponding *N. meningitidis porA* sequence.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The present invention is directed to improving nucleic acid-based detection of
5 *N. gonorrhoeae*. To this end the present inventors have identified an alternative PCR target sequence on the *N. gonorrhoeae* genome, namely the *porA* pseudogene, which has never before been used as a target for *N. gonorrhoeae* detection and unexpectedly offers drastically improved clinical sensitivity and specificity for the detection of *N. gonorrhoeae* when compared with other PCR assays described to
10 date.

More particularly, the present inventors have developed a new *N. gonorrhoeae* LightCycler™ assay targeting the *N. gonorrhoeae porA* pseudogene. Importantly, the *Neisseria porA* gene is known to exist only in pathogenic *Neisseria*, comprising *N. gonorrhoeae* and *N. meningitidis*, and is not found in the commensal
15 *Neisseria* species (Feavers et al 1998; Perrin et al 1999). Therefore, the selection of this gene target eliminates or at least reduces the potential for cross-reaction with the commensal *Neisseria* species. Differences existing in *porA* sequences between *N. gonorrhoeae* and *N. meningitidis* have also been exploited to develop oligonucleotides that enable specific PCR amplification and detection of *N.*
20 *gonorrhoeae*-derived nucleic acids only.

The present invention therefore provides methods for detecting an isolated *porA* nucleic acid of *Neisseria gonorrhoeae* in a biological sample and one or more

oligonucleotides, which facilitate amplification and/or detection of said *por A* nucleic acid.

For the purposes of this invention, by "*isolated*" is meant material that has been removed from its natural state or otherwise been subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. Isolated material may be in native, chemical synthetic or recombinant form.

As used herein, "*nucleic acid*" includes and encompasses DNA, RNA and DNA-RNA hybrids. DNA includes single-stranded and double-stranded genomic DNA and cDNA as are well understood in the art. RNA includes single-stranded and double-stranded unprocessed RNA, mRNA and tRNA.

It will be appreciated that by "*porA* nucleic acid" is meant a nucleic acid which corresponds to at least a fragment or region of a *porA* gene or pseudogene.

As used herein, a "*gene*" is a discrete structural unit of a genome which may comprise one or more elements such as an amino acid coding region typically present in one or more cistrons, an operator, a promoter, a terminator and/or any other regulatory nucleotide sequence(s).

As used herein a "*pseudogene*" is an inactive unit, region or sequence of a genome which has a similar sequence to a known functional gene. Typically, because of this sequence similarity, pseudogenes are normally considered to be evolutionary relatives to normally functioning genes.

In *N. gonorrhoeae* *porA* is a pseudogene while in *N. meningitidis* *porA* is a gene.

By "corresponds to" or "corresponding to" in this context is meant that the *porA* nucleic acid comprises a nucleotide sequence which is present in an *porA* pseudogene, or is complementary to a nucleotide sequence present in a *porA* pseudogene, or is at least 80%, preferably at least 85%, more preferably at least 90% or even more preferably at least 95%, 96%, 97%, 98% or 99% identical to either of these.

In a particularly preferred embodiment the *porA* nucleic acid corresponds to a 132 bp fragment of a *porA* pseudogene.

In particular aspects, the invention provides one or more oligonucleotides and/or methods of using same for facilitating nucleic acid sequence amplification and/or detection of a *porA* nucleic acid.

As used herein, an "oligonucleotide" is a single- or double-stranded nucleic acid having no more than one hundred (100) nucleotides (bases) or nucleotide pairs (base pairs). A "polynucleotide" has more than one hundred (100) nucleotides or nucleotide pairs.

In the particular context of nucleic acid sequence amplification, an oligonucleotide of the invention may be in the form of a primer.

As used herein, a "primer" is a single-stranded oligonucleotide which is capable of hybridizing to a nucleic acid "template" and being extended in a template-dependent fashion by the action of a suitable DNA polymerase such as *Taq* polymerase, RNA-dependent DNA polymerase or SequenaseTM.

Typically, a primer may have at least twelve, fifteen, twenty, twenty-five, thirty, thirty five or forty but no more than fifty contiguous nucleotide bases.

It will be appreciated that the primers described herein have been designed according to selected criteria to maximize detection sensitivity and specificity.

5 Suitably, primers of the invention are designed to be capable of annealing to a nucleotide sequence of a *Neisseria gonorrhoeae porA* nucleic acid that is not present in, or has one or more nucleotides different to, a nucleotide sequence of a *porA* nucleic acid of another *Neisseria* species, such that said oligonucleotide is not capable of annealing to said *porA* nucleic acid of said another *Neisseria* species.
10 sufficient to facilitate detection of said *porA* nucleic acid of said another *Neisseria* species.

Preferably, said another *Neisseria* species is *N. meningitidis*.

It will also be appreciated that the present invention is predicated, in part, on the observatin that other, commensal *Neisseria* species such as *N. subflava*, *N.*
15 *cinerea*, *N. flavescens*, *N. lactamica* and *N. sicca* do not have a *porA* pseudogene.

In one particularly advantageous embodiment, primers of the invention are designed to facilitate detection of a *porA* nucleic acid of a plurality of isolates, strains, allelic variants or sub-species of *Neisseria gonorrhoeae*.

Accordingly, the present inventors have identified nucleotide sequences in a
20 *porA* gene of *Neisseria gonorrhoeae*, which are conserved within a plurality of isolates of this pathogenic organism, which sequences are not present in, or are sufficiently different to, respective nucleotide sequences in a *porA* pseudogene of *Neisseria meningitidis*.

This has enabled the present inventors to design primers that facilitate specific, sensitive and broad-spectrum amplification of *Neisseria gonorrhoeae porA* nucleic acids while avoiding amplification of a *porA* nucleic acid of said another *Neisseria* species, or at least minimizing amplification thereof to an undetectable level.

Particular examples of primers according to the invention are provided in FIG. 1 and Table 1.

It will be appreciated from comparing the primer sequences of FIG. 1, the *N. gonorrhoeae porA* pseudogene nucleotide sequence set forth in FIG.1 and the corresponding published *N. meningitidis porA* nucleotide sequence, that variations in primer sequence are readily achievable while maintaining the specificity necessary for selectively amplifying a *N. gonorrhoeae porA* sequence.

In a preferred embodiment the invention contemplates detection of a *porA* nucleic acid or fragment thereof by nucleic acid sequence amplification and subsequent detection of a *porA* amplification product

Nucleic acid amplification techniques are well known to the skilled addressee, and include polymerase chain reaction (PCR) and ligase chain reaction (LCR) as for example described in Chapter 15 of Ausubel *et al.* CURRENT PROTOCOLS IN MOLECULAR BIOLOGY (John Wiley & Sons NY, 1995-1999); strand displacement amplification (SDA) as for example described in U.S. Patent No 5,422,252; rolling circle replication (RCR) as for example described in Liu *et al.*, 1996, J. Am. Chem. Soc. 118 1587 and International application WO 92/01813 and by Lizardi *et al.*, in International Application WO 97/19193; nucleic acid sequence-

based amplification (NASBA) as for example described by Sooknanan *et al.*, 1994, Biotechniques 17 1077; and Q- β replicase amplification as for example described by Tyagi *et al.*, 1996, Proc. Natl. Acad. Sci. USA 93 5395.

The abovementioned are examples of nucleic acid sequence amplification
5 techniques but are not presented as an exhaustive list of techniques. Persons skilled in the art will be well aware of a variety of other applicable techniques as well as variations and modifications to the techniques described herein.

For example, the invention contemplates use of particular techniques that facilitate quantification of nucleic acid sequence amplification products such as by
10 "competitive PCR", or techniques such as "Real-Time" PCR amplification such as described in Whiley *et al.*, 2002, Bacteriology 42 85.

Preferably, the nucleic acid sequence amplification technique is PCR.

As used herein, an "*amplification product*" is a nucleic acid generated by a nucleic acid sequence amplification technique as hereinbefore described.

15 In a particularly preferred embodiment the method produces a single 132 bp *porA* amplification product.

As used herein, "*hybridization*", "*hybridize*" and "*hybridizing*" refers to formation of a hybrid nucleic acid through base-pairing between complementary or at least partially complementary nucleotide sequences under defined conditions, as is
20 well known in the art. Normal base-pairing occurs through formation of hydrogen bonds between complementary A and T or U bases, and between G and C bases. It will also be appreciated that base-pairing may occur between various derivatives of purines (G and A) and pyrimidines (C, T and U). Purine derivatives include inosine,

methylinosine and methyladenosines. Pyrimidine derivatives include sulfur-containing pyrimidines such as thiouridine and methylated pyrimidines such as methylcytosine. For a detailed discussion of the factors that generally affect nucleic acid hybridization, the skilled addressee is directed to Chapter 2 of CURRENT
5 PROTOCOLS IN MOLECULAR BIOLOGY, *supra*.

More specifically, the terms "*anneal*" and "*annealing*" are used in the context of primer hybridization to a nucleic acid template for a subsequent primer extension reaction, such as occurs during nucleic acid sequence amplification or dideoxy nucleotide sequencing, for example.

10 For a discussion of the factors that affect annealing of PCR primers, the skilled addressee is directed to Chapter 15 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds Ausubel et al. (John Wiley & Sons NY 1995-1999).

The invention provides detection of a *porA* nucleic acid of *Neisseria gonorrhoeae* in a biological sample as an indication or the presence of a *Neisseria*
15 *gonorrhoeae* in an individual from which the biological sample has been derived or obtained, or as an indication of a past *Neisseria gonorrhoeae* infection of said individual.

Preferably, a *porA* nucleic acid amplification product, which amplification product is detected by one or more methods as well understood in the art.

20 Detection of amplification products may be achieved by detection of a probe hybridized to an amplification product, by direct visualization of amplification products by way of agarose gel electrophoresis, nucleotide sequencing of

amplification products or by detection of fluorescently-labeled amplification products.

As used herein, a "probe" is a single- or double-stranded oligonucleotide or polynucleotide, one and/or the other strand of which is capable of hybridizing to another nucleic acid, to thereby form a "hybrid" nucleic acid

Probes and/or primers of the invention may be labeled, for example, with biotin or digoxigenin, with fluorochromes or donor fluorophores such as FITC, TRITC, Texas Red, TET, FAM6, HEX, ROX or Oregon Green, acceptor fluorophores such as LC-Red640, enzymes such as horseradish peroxidase (HRP) or alkaline phosphatase (AP) or with radionuclides such as ^{125}I , ^{32}P , ^{33}P or ^{35}S to assist detection of amplification products by techniques are well known in the art.

Particularly preferred embodiments of probes according to the present invention are set forth in FIG. 1 and Table 2.

With regard to detection of fluorescently-labeled amplification products, this may be achieved using one or more primers that incorporate fluorescent labels as hereinbefore described.

In another embodiment, detection may be performed by melting curve analysis using probes incorporating fluorescent labels that hybridize to amplification products in a sequence amplification reaction. A particular example is the use of Fluorescent Resonance Energy Transfer (FRET) probes to hybridize with amplification products in "real time" as amplification products are produced with each cycle of amplification.

In yet another embodiment, the invention contemplates use of melting curve analysis whereby nucleic acid-intercalating dyes such as ethidium bromide (EtBr) or SYBR Green I bind amplification products and fluorescence emission by the intercalated complexes is detected.

5 Melting curve analysis may advantageously be performed using an apparatus such as a LightCycler™, as for example described in Whiley *et al.*, 2002, *supra*.

In light of the foregoing, it will be apparent that the *Neisseria gonorrhoeae* detection methods of the invention are ideally suited to assisting diagnosis of individuals that may have had, or currently have, a *Neisseria gonorrhoeae* infection.

10 *Neisseria gonorrhoeae* is a primarily a pathogenic organism of humans, hence the present invention is particularly directed to detection of *Neisseria gonorrhoeae* infection in human individuals.

Suitably, said biological sample is a cervical, urethral, penile, anal, rectal, throat, saliva, fecal or urine sample, although without limitation thereto.

15 Suitably, said biological sample includes one ore more bacteria or nucleic acid(s) derived therefrom which may be in the form of DNA or RNA.

Preferably, in order to minimize handling of said biological sample, genomic DNA is isolated from said biological sample according to the method of the invention. However, in principle, cDNA could be generated by reverse-transcribing
20 isolated RNA as is well known in the art, as for example described in Chapter 15 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, *supra*.

Particularly for the purpose of clinical diagnosis, although without limitation thereto, the invention provides a kit comprising one or more oligonucleotides such as

a primer pair according to the third aspect of the invention, Said kit may further comprise other reagents such as a thermostable DNA polymerase, a *porA* nucleic acid probe, positive and/or negative nucleic acid control samples, molecular weight markers, detection reagents such as for colorimetric detection or fluorescence
5 detection of amplification products and/or reaction vessels such as microtitre plates.

It will also be appreciated that the method of the invention may be used alone or combined with other forms of diagnosis, such as bacterial culture tests or traditional diagnosis based on clinical symptoms, to improve the accuracy of diagnosis.

10 In a preferred embodiment the invention provides a method of determining whether a human individual is or has been infected with *Neisseria gonorrhoeae*, said method including the steps of:

(i) subjecting a biological sample obtained from said human individual to nucleic acid sequence amplification to selectively produce a *porA* *Neisseria*
15 *gonorrhoeae* amplification product from a *Neisseria gonorrhoeae porA* nucleic acid if present in said biological sample; and

(ii) detecting said amplification product, if present, by probe hybridization whereby a presence of said *porA* amplification product indicates that said individual is or has been infected with *Neisseria gonorrhoeae*.

20 According to a particularly preferred form of this embodiment, real-time PCR detection is utilized at step (ii), by fluorescence resonance energy transfer (FRET) using an adjacent hybridization probe format. The preferred upstream oligonucleotide probe (papPI; Table 1) is labelled with a donor fluorophore,

fluorescein, at the 3' terminus, and the preferred downstream oligonucleotide probe (papP2; Table 1) is labelled with an acceptor fluorophore, LC-Red640, at the 5' terminus. Probe papP2 is also phosphorylated at the 3' terminus. T

It will be further appreciated that the present invention may be performed in
5 conjunction with nucleic acid-based detection of other pathogenic organisms.

In this regard, the invention contemplates nucleic acid array detection of a *porA* nucleic acid of *Neisseria gonorrhoeae*, wherein one or more other nucleic acids of other pathogenic organisms may be detected.

A general discussion of this type of microarray approach is provided in
10 Bryant *et al.*, 2004, Lancet Infect. Dis. 4 100.

It will be appreciated that the nucleic acid array may comprise an oligonucleotide according to the second aspect, immobilized, coupled, impregnated or otherwise in communication with a substrate.

More generally, nucleic acid array technology has become well known in the
15 art and examples of methods applicable to array technology are provided in Chapter 22 of CURRENT PROTOCOLS IN MOLECULAR BIOLOGY Eds. Ausubel *et al.* (John Wiley & Sons NY USA 1995-2001).

In certain embodiments, at least one address of the plurality includes a nucleic acid capture probe that hybridizes specifically to a member of a nucleic acid
20 library, *e.g.*, the sense or anti-sense strand. In one preferred embodiment, a subset of addresses of the plurality of addresses has a nucleic acid capture probe for a nucleic acid library member. Each address of the subset can include a capture probe that hybridizes to a different region of a library member.

With respect to the present invention, a preferred array format comprises glass slides having an immobilized, ordered grid of a plurality of cDNA fragments.

The array can have a density of at least than 10, 50, 100, 200, 500, 1,000, 2,000, or 10,000 or more addresses/cm², and ranges therebetween. The substrate
5 may be a two-dimensional substrate such as a glass slide, a wafer (e.g., silica or plastic), a mass spectroscopy plate, or a three-dimensional substrate such as a gel pad.

An array can be generated by various methods, e.g., by photolithographic methods (see, e.g., U.S. Patent Nos. 5,143,854; 5,510,270; and 5,527,681),
10 mechanical methods (e.g., directed-flow methods as described in U.S. Patent No. 5,384,261), pin-based methods (e.g., as described in U.S. Pat. No. 5,288,514), and bead-based techniques (e.g., as described in PCT/US93/04145).

So that the present invention can be readily understood and put into practical effect, reference is made to the following non-limiting examples.

15

EXAMPLES

MATERIALS & METHODS

Patient specimens

A total of 282 clinical samples (46 cervical swabs, 12 urethral swabs and 224 urine specimens) from patients presenting for sexual health screen were used in this
20 study. Given the low incidence of *N. gonorrhoeae* infection in our local population, the samples were selected to provide a large proportion of Cobas positive specimens.

The patients comprised 178 females and 104 males and ranged in age from 13 to 70 years with a mean age of 26 years and a median age of 23 years. All 282

samples were tested by the NGpapLC assay and by a testing algorithm combining the Roche Cobas Amplicor assay and the cppB-LC assay. Using this algorithm, specimens were initially tested by the Cobas Amplicor method. Any sample providing a positive *N. gonorrhoeae* result by the Cobas Amplicor assay was then
5 tested by the cppB-LC assay for confirmation.

Roche Cobas Amplicor Assay

The urine and swab specimens were processed and tested on the Roche Cobas Amplicor System according to the manufacturer's instructions. Each specimen was simultaneously tested for *N. gonorrhoeae*, *Chlamydia trachomatis* and the presence
10 of inhibiting substances. Result interpretation was performed using the criteria supplied by the manufacturer. Briefly, specimens providing an absorbance less than 0.2 were considered negative for *N. gonorrhoeae* whereas specimens providing an absorbance value of 0.2 or greater were considered positive.

LightCycler assays (NGpapLC and cppB-LC)

15 A column extraction was used for both the NGpapLC and cppB-LC LightCycler assays. Nucleic acids were extracted from 0.2 ml of each specimen using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Australia), according to the manufacturer's instructions. Purified specimen DNA was eluted from the column in 50 µl of elution buffer (Roche Diagnostics, Australia). DNA extracts were stored at –
20 20°C until analysis.

The NGpapLC assay comprised of one primer pair, one pair of hybridization probes and targeted the *N. gonorrhoeae* *porA* pseudogene. Amplification was performed using primers papF and papR (Table 1; Invitrogen, Australia), which

produced a 132 bp PCR product during the reaction. Real-time PCR detection was achieved by fluorescence resonance energy transfer (FRET) using an adjacent hybridization probe format. The upstream oligonucleotide probe (papP1; Table 1) was labelled with a donor fluorophore, fluorescein, at the 3' terminus, and the downstream oligonucleotide probe (papP2; Table 1) was labelled with an acceptor fluorophore, LC-Red640, at the 5' terminus (TIBMOBBIOL, Germany). Probe papP2 was also phosphorylated at the 3' terminus. The LightCycler FastStart DNA Master Hybridization Probes kit (Roche Diagnostics, Australia) was used as the basis for the reaction mixture, employing a 20µl volume in each reaction capillary.

5 Briefly, capillaries were loaded with 2µl of kit Master reagent (10 x; Roche Diagnostics, Australia, reagent 1), 4mM of MgCl₂ (Roche Diagnostics, Australia, reagent 2), 0.4µM of primer papF, 0.6µM of primer papR, 0.2µM of each hybridization probe and 5µl of DNA extract. Each mix was made up to 20µl using sterile PCR-grade water (Roche Diagnostics, Australia, reagent 3). Every test run

15 included a positive control and three no-target controls consisting of 15 µl of reaction mixture with 5µl of extraction elution buffer. Amplification and detection was performed on the LightCycler (software version 5.32) using the following parameters: an initial denaturation step at 95°C for 10-minutes followed by 55 cycles of denaturation at 95°C for 10 seconds, primer and probe annealing at 55°C for 10

20 seconds and extension at 72°C for 20 seconds. The fluorescence response data were obtained during the annealing period and analysed with the channel settings F2/F1. Melting curve analysis was performed following PCR amplification. Briefly, the

analysis was commenced at 40°C, and the temperature was raised to 95°C at a rate of 0.2°C/s.

The cppB-LC assay was performed as previously described (Whiley et al 2002) and utilized similar conditions to those of the NGpapLC assay. Briefly, the
 5 LightCycler FastStart DNA Master Hybridisation Probes kit (Roche Diagnostics, Australia) was used as the basis of the reaction mix. Each reaction capillary comprised a total reaction volume of 20µl, which included two primers (HO1 @ 0.2 µM and HO2 @ 0.4µM) and two hybridisation probes (0.2µM each) targeting the *N. gonorrhoeae* cppB gene (Whiley et al 2002).

10 *Detection limit*

The detection limits of the NGpapLC and cppB-LC assays were determined and compared by testing dilutions of a suspension of a *N. gonorrhoeae* culture (ATCC49226) at 5 x 10E4 colony forming units/ml (cfu/ml). Serial 10-fold dilutions of this suspension were extracted and tested by both assays using the conditions
 15 described above. The detection limit of each assay was determined as the lowest concentration returning a positive reaction.

Neisseria panel

A panel of *Neisseria* species was tested by both the NGpapLC and cppB-LC assays. This panel included 63 non-gonococcal *Neisseria* isolates, which were tested
 20 to determine false-positive cross-reactions. The species comprised *N. meningitidis* (38), *N. subflava* (12), *N. sicca* (6), *N. elongata* (3), *N. mucosa* (1), *N. lactamica* (3) and *Branhamella catarrhalis* (6), which is closely related to the *Neisseria* genus. In addition, 84 *N. gonorrhoeae* isolates were tested to determine if the primer and probe

sequence targets of each assay were conserved. Six *N. gonorrhoeae* isolates were included that had previously tested negative by the cppB-LC assay. These six isolates were kindly provided by Royal Darwin Hospital, Northern Territory. The remaining *N. gonorrhoeae* isolates were selected from different geographical locations throughout the state of Queensland to ensure a broad cross section of isolates. Nucleic acids were extracted from cultures of each isolate using the High Pure Viral Nucleic Acid kit (Roche Diagnostics, Australia), according to the manufacturer's instructions. Approximately 0.5 µg of bacterial DNA was added to each reaction.

Non-Neisseria panel – common human pathogens and normal flora

10 An additional panel of common human pathogens and normal flora was also used to further determine the specificity of the NGpapLC assay; *Acinetobacter anitratus* ATCC 19606, *Acinetobacter baumannii* ACM 686, *Acinetobacter haemolyticus* ACM 620, *Acinetobacter johnsonii* ACM 621, *Acinetobacter junii* ACM 617, *Acinetobacter lwoffii* ACM 664, *Aeromonas hydrophilia* ATCC 35654, 15 *Alcaligenes faecalis* ATCC 35655, *Bacillus amyloliquifaciens* ATCC 3642, *Bacillus brevis* ATCC 37, *Bacillus cereus* ATCC 11778, *Bacillus circulans* ATCC 61, *Bacillus coagulans* ATCC 264, *Bacillus firmus* ATCC 31, *Bacillus laterosporus* ATCC 295, *Bacillus licheniformis* ATCC 127559, *Bacillus macerans* ATCC 401, *Bacillus megaterium* ATCC 2640, *Bacillus mycoides* ATCC 28, *Bacillus polymyxa* 20 ATCC 35, *Bacillus pumilis* ATCC 433, *Bacillus sphaericus* ATCC 4525, *Bacillus subtilis* ATCC 11774, *Bacillus thuringiensis* ATCC 453, *Bacteroides distasonis* ATCC 8503, *Bacteroides gingivalis* ATCC 33277, *Bacteroides vulgatus* ATCC 8482, *Bordetella bronchiseptica* ATCC 10580, *Bordetella parapertussis* ATCC

- 15237, *Burkholderia cepacia* ATCC 17765, *Campylobacter jejuni* ATCC 33291, *Candida albicans* ATCC 14053, *Candida krusei* (laboratory isolate), *Candida tropicalis* (laboratory isolate), *Citrobacter freundii* ATCC 8090, *Corynebacterium diphtheriae* ATCC 13812, *Enterobacter aerogenes* ATCC 13048, *Enterobacter*
- 5 *cloacae* (laboratory isolate), *Enterococcus durans* ATCC 6506, *Enterococcus faecalis* ATCC 29212, *Enterococcus faecium* ATCC 35667, *Erysipelothrix rhusiopathiae* ATCC 19414, *Escherichia coli* ATCC 35218, *Flavobacterium indologenes* (laboratory isolate), *Flavobacterium multivoram* ATCC 35656, *Haemophilis influenzae* ATCC 10211, *Klebsiella pneumoniae* ATCC 13883, *Listeria*
- 10 *monocytogenes* ATCC 7646, *Micrococcus luteus* ATCC 4988, *Proteus mirabilis* ATCC 7002, *Proteus vulgaris* ATCC 6380, *Providencia stuartii* ATCC 35031, *Pseudomonas aeruginosa* ATCC 27853, *Pseudomonas vesicularis* (laboratory isolate), *Saccharomyces cerevisiae* ATCC 2366, *Salmonella typhimurium* ATCC 14028, *Serratia marcescens* (laboratory isolate), *Serratia oderifera* ATCC 33077,
- 15 *Shigella flexneri* (laboratory isolate), *Shigella sonnei* ATCC 25931, *Staphylococcus simulans* ATCC 27851, *Staphylococcus aureus* (laboratory isolate), *Staphylococcus aureus* ATCC 33591, *Staphylococcus aureus* NCTC 6751, *Staphylococcus capitis* ATCC 27840, *Staphylococcus cohnii* ATCC 29974, *Staphylococcus epidermidis* (laboratory isolate), *Staphylococcus haemolyticus* ATCC 29970, *Staphylococcus*
- 20 *hominus* ATCC 27844, *Staphylococcus intermedius* ATCC 29663, *Staphylococcus lugdenensis* (laboratory isolate), *Staphylococcus scuri* (laboratory isolate), *Staphylococcus warneri* ATCC 27836, *Staphylococcus xylosus* ATCC 29971, *Stenotrophomonas maltophilia* (laboratory isolate), *Streptococcus agalactiae* ATCC

12386, *Streptococcus bovis* ATCC 9809, *Streptococcus equi* ATCC 9528, *Streptococcus equisimilis* ATCC 35666, *Streptococcus* (group B) ATCC 12386, *Streptococcus* (group F) ATCC 12392, *Streptococcus* (group G) ATCC 12394, *Streptococcus mutans* ATCC 35668, *Streptococcus pneumoniae* ATCC 27336, 5 *Streptococcus pyogenes* ATCC19615, *Streptococcus salivarius* ATCC13419, *Vibrio alginolyticus* ATCC17749, *Vibrio parahaemolyticus* ATCC17802, *Yarrowia lipolytica* ATCC 9773 and *Yersinia enterocolitica* ATCC 23715. Genomic DNA was purified from cultures of these organisms and tested in the NGpapLC assay using the conditions described above.

10

RESULTS

A total of 282 clinical samples were tested by the NGpapLC assay and by a testing algorithm combining the Roche Cobas Amplicor assay with the cppB-LC assay. A summary of these results is provided in Table 2.

Overall, 79 (28.0%) specimens were positive and 120 (42.6%) specimens 15 were negative for *N. gonorrhoeae* DNA by all three PCR methods. An additional 81 (28.7%) specimens were positive by the Cobas Amplicor assay but negative by both the NGpapLC and cppB-LC assays. These were considered to be false-positive results obtained by the Cobas Amplicor. A further two (0.7%) specimens were positive by the Cobas Amplicor assay but gave discrepant results on the LightCycler 20 assays; one specimen was positive by the cppB-LC assay with a cycle threshold value (Ct value) of 47 but negative by NGpapLC whereas the remaining specimen was positive by NGpapLC only, providing a Ct value of 41. These Ct values were the highest recorded Ct values for each test and are indicative of low *N. gonorrhoeae*

DNA concentrations in each specimen. Upon repeat testing, neither specimen was consistently positive. This suggests that the concentration of DNA in both specimens was on the threshold of sensitivity of the respective test.

By testing dilutions of a *N. gonorrhoeae* culture, ranging from 5×10^4 to 5×10^{-2} cfu/ml, the limit of sensitivity of the NGpapLC was determined to be 5 cfu/ml of *N. gonorrhoeae* in the specimen. The detection limit of the cppB-LC was determined to be 5×10^{-1} cfu/ml. This suggests the cppB-LC has a 10-fold better detection limit than the NGpapLC assay.

To further determine the specificity of the NGpapLC assay, genomic DNA was purified and tested from cultures of a broad panel of organisms. These included *Neisseria* species as well as other common human pathogens and normal flora. All of the non-gonococcal species were negative when tested by the NGpapLC assay. In contrast, three *N. meningitidis* isolates tested positive by the cppB-LC assay. These three *N. meningitidis* isolates provided cycle threshold (Ct) values in the cppB-LC assay that were significantly greater than those provided by the positive *N. gonorrhoeae* isolates; the Ct values of the *N. gonorrhoeae* isolates were consistently lower than 16 whereas these three *N. meningitidis* isolates provided Ct values of 33 or greater. This suggests the cppB gene may be present at lower copy number in these *N. meningitidis* isolates.

Of the 84 *N. gonorrhoeae* isolates tested, seven were negative by the cppB-LC assay. Six of these seven negative isolates were the *N. gonorrhoeae* isolates obtained from the Northern Territory and had previously tested negative by the cppB-LC assay. Therefore only one additional cppB negative isolate was identified

in the Queensland isolates. In contrast, all 84 *N. gonorrhoeae* isolates provided positive results when tested by the NGpapLC assay. This shows that the NGpapLC oligonucleotide targets were present in all isolates. In addition, fluorescent melting curve analysis by the NGpapLC assay showed no variation between *N. gonorrhoeae* isolates or any of the positive clinical specimens; all NGpapLC positive reactions provided melting temperatures of 65°C.

NGpapLC compared to bacterial culture

A comparison with bacterial culture was performed on the local Queensland population and comprised of 557 specimens taken from patients attending sexual health clinics. This population was considered ideal for testing the specificity of the NGpapLC assay given the low incidence of *N. gonorrhoeae* in this population.

Compared to bacterial culture, the assay was 100% sensitive and 88.2% specific (Table 3).

However, this specificity calculation is based on the assumption that the two additional PCR positives are false-positive results. In contrast, we believe that these additional positive results are true positive results. This is because the results of the NGpapLC were supported by a second PCR assay targeting a separate *N. gonorrhoeae* gene. In addition, previous studies have shown that PCR has better clinical sensitivity than bacterial culture. Therefore, additional PCR positives would not be unexpected. Overall, the above results suggest the new NGpapLC assay is highly suitable for routine detection of *N. gonorrhoeae* in clinical samples.

Tables 4-7 provide a breakdown of the Table 3 data into specimen types.

For both the urethral and cervical specimens the NGpapLC assay was 100% specific.

The results of the throat swabs suggest the NGpapLC is also suitable for use on these specimen types, as does the more limited data on anal swabs.

5

DISCUSSION

The problems associated with the specificity of the Cobas Amplicor *N. gonorrhoeae* assay and the requirements for a confirmatory assay are well documented. Unfortunately, the conventional gene targets used for confirmatory tests have also proved to have limitations. The results of this study suggest the NGpapLC
10 assay is a suitable alternative to the cppB-LC for confirmation of Cobas Amplicor *N. gonorrhoeae* positive results. By targeting the *N. gonorrhoeae* porA pseudogene, the NGpapLC overcomes the limitations associated with the cppB gene and provides the potential for improved clinical sensitivity and specificity.

By testing dilutions of an ATCC strain of *N. gonorrhoeae*, the cppB-LC assay
15 had a 10-fold better detection limit compared to our new NGpapLC assay. This presumably is because the cryptic plasmid is at a higher copy number than the *N. gonorrhoeae* porA pseudogene. Nevertheless, the difference in detection limits did not affect the clinical sensitivities of the assays. For the clinical specimens, the NGpapLC and cppB-LC assays gave good agreement for the detection of *N.*
20 *gonorrhoeae*. Only two of the 282 specimens provided discrepant results, with each LightCycler assay detecting an additional positive over the other. Originally, it was considered that the additional cppB-LC positive result may have stemmed from the *N. gonorrhoeae* DNA load being below the detection limit of the NGpapLC assay.

However, by using a more sensitive nested PCR assay we were still unable to detect the presence of the *N. gonorrhoeae* porA pseudogene in this specimen (data not shown). This suggests that this specimen was either a false-positive using our Roche Amplicor and cppB-LC testing algorithm or represents a *N. gonorrhoeae* strain lacking the porA pseudogene. Although, to date there have been no reports of *N. gonorrhoeae* strains lacking the porA pseudogene. It is further interesting to note that by using nested amplification we were able to detect the presence of the cppB gene in the specimen that was negative by the cppB-LC assay but positive by the NGpapLC test. This demonstrates that this cppB-LC negative result did not occur because of the absence of the cppB gene in this presumptive *N. gonorrhoeae* strain (data not shown).

Overall, the results for the clinical specimens suggest the NGpapLC and cppB-LC assays have similar clinical sensitivities and specificities and that the cppB-LC assay may in fact be suitable for use on urine and genital swab specimens in our population. However, these results are in contrast with those of the bacterial panel, which highlighted the limitations of the cppB-LC assay. Most notably the cppB-LC assay failed to detect seven *N. gonorrhoeae* isolates. This shows that in our population there are *N. gonorrhoeae* strains lacking the cppB gene. More importantly, our testing algorithm is likely to produce false-negative results; specimens testing positive by the Cobas Amplicor could incorrectly be identified as false-positives by the cppB-LC assay. In this study, *N. gonorrhoeae* isolates were not randomly selected therefore more testing is required to determine the overall cppB-LC false-negative rate. Other studies have suggested that the incidence of such

isolates lacking the *cppB* gene is low in Australia (Leslie et al 2003). Nevertheless, when using the *cppB*-LC assay the potential for false-negative results exists. In contrast, the NGpapLC method correctly identified all *N. gonorrhoeae* isolates tested in this study. This suggests the NGpapLC assay may not be susceptible to false-
5 negative results arising from the absence of the *porA* pseudogene in our local *N. gonorrhoeae* strains. Hence, it should provide improved clinical sensitivity compared to the *cppB*-LC assay.

The NGpapLC assay also proved to be highly specific for *N. gonorrhoeae* DNA; all non-gonococcal species provided negative results when tested by the
10 NGpapLC method. It should be noted that we experienced difficulty obtaining *Neisseria* species and so our bacterial panel did not include all known *Neisseria* species and only contained a limited number of isolates for most species. Therefore, cross-reactions with other *Neisseria* species cannot be ruled out on the basis of these results alone. However, the *Neisseria porA* gene is only reported to exist in *N.*
15 *gonorrhoeae* and *N. meningitidis* (Feavers et al 1998), and therefore cross-reactions with *N. meningitidis* were considered to be more relevant. In this study, we tested 38 *N. meningitidis* isolates and found no cross-reactions. As a result, we are confident of the specificity of this test when used on our local sample population.

The *cppB*-LC results for the bacterial panel provided a good example of the
20 specificity problems associated with using the *cppB* target as positive results were obtained from three *N. meningitidis* cultures. Nevertheless, the presence of the *cppB* gene in local *N. meningitidis* isolates may not pose a major specificity problem if testing is restricted to urine and genital swab specimens. To date there have been no

reports of *N. meningitidis* producing false-positive results in the Cobas Amplicor assay, and such isolates would be negative when initially screened by the Cobas Amplicor. On the other hand, the *cppB* gene has been found in isolates of other *Neisseria* species, including *N. cinerea*, which also cross-reacts with the Cobas Amplicor assay (Palmer et al 2003; Farrell 1999). Further, our study only examined a limited number of *Neisseria* species and strains, therefore, the possibility of cross-reactions with other *Neisseria* species in our population cannot be excluded.

The importance surrounding the specificity of a confirmatory assay for *N. gonorrhoeae* is also dependent on specimen site. One of the key limitations of the Cobas Amplicor is that its specificity significantly declines when used on extra-genital sites. In particular, throat swabs are a major problem as they can possess various *Neisseria* species and so offer greater potential for false-positive cross-reactions. A recent study by Leslie et al (2003) found that confirmation rates of Cobas Amplicor *N. gonorrhoeae* positive results dropped from 86.2% for penile and urethral swabs to 5.6% for oropharyngeal swabs. It should be noted that even when using the *cppB*-LC confirmatory assay, the number of true positives obtained from throat swabs are likely to be much lower than those obtained from genital specimens. This is because the presence of multiple *Neisseria* species in throat swabs increases the chance of both assays providing false-positive results; one species could cross-react with the screening assay while another species may cross-react with the confirmatory assay, thus producing a false-positive result from the algorithm. We believe that the specificity provided by our new NGpapLC confirmatory assay may provide improved detection of *N. gonorrhoeae* in extra-genital sites. Our aim is to

extensively evaluate the use of the NGpapLC assay on multiple specimen types, including throat swabs.

The results of this study show the *porA* pseudogene is a suitable target for PCR detection of *N. gonorrhoeae*. It is worth highlighting that the *porA* gene was previously a popular target for the detection of *N. meningitidis* by PCR. However, it has since lost favour since it was discovered that insertion sequences may be incorporated into the *porA* gene of some *N. meningitidis* isolates giving false-negative results (van der Ende et al. 1999; Newcombe et al. 1998; Jelfs et al. 2000). Such mutations can potentially block PCR amplification or probe hybridization and hence prevent detection of some *N. meningitidis* isolates. The fact that all *N. gonorrhoeae* isolates were detected in this study suggests that insertion sequences may not be found in the *N. gonorrhoeae* *porA* pseudogene. However, given that the selection of our *N. gonorrhoeae* isolates was not random, it is possible that *N. gonorrhoeae* isolates containing insertion sequences were simply missed by the study. Alternatively, insertion sequences may have been present but did not affect the NGpapLC assay because of its small PCR product size (132bp); such a small PCR product size may reduce the probability of sequences inserting between the PCR primer targets. We aim to further evaluate the NGpapLC assay against an extended panel of *N. gonorrhoeae* isolates.

Throughout the specification the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one embodiment or specific collection of features. It will therefore be appreciated by those of skill in the art that, in light of the instant disclosure, various modifications and changes can

be made in the particular embodiments exemplified without departing from the scope of the present invention.

All patent and scientific literature, algorithms and computer programs referred to in this specification are incorporated herein by reference in their entirety.

REFERENCES

- Leslie DE, Azzato F, Ryan N, Fyfe J. An assessment of the Roche Amplicor Chlamydia trachomatis/Neisseria gonorrhoeae multiplex PCR assay in routine
 5 diagnostic use on a variety of specimen types. Commun Dis Intell. 2003;27(3):373-9.
- Feavers IM, Maiden MC. A gonococcal porA pseudogene: implications for understanding the evolution and pathogenicity of Neisseria gonorrhoeae Mol.
 10 Microbiol. 1998 30(3):647-656.
- Perrin A, Nassif X, Tinsley C. Identification of regions of the chromosome of Neisseria meningitidis and Neisseria gonorrhoeae which are specific to the pathogenic Neisseria species. *Infect Immun*. 1999 Nov;67(11):6119-29.
 15
- Ottawa: A cluster of culture-positive, but PCR false negative infections with Neisseria gonorrhoeae. John Tapsall, Athena Limnios, Ian Carter, Gary Lum, Kevin Freeman, Theo Sloots. Abstract 0129. 15th Biennial Congress of the International Society for Sexually Transmitted Diseases Research (ISSTD).
- 20
- Palmer HM, Mallinson H, Wood RL, Herring AJ. Evaluation of the specificities of five DNA amplification methods for the detection of Neisseria gonorrhoeae. J Clin Microbiol. 2003 Feb;41(2):835-7.

- Farrell DJ.** Evaluation of AMPLICOR *Neisseria gonorrhoeae* PCR using *cppB* nested PCR and 16S rRNA PCR. *J Clin Microbiol.* 1999 Feb;37(2):386-90.
- 5 **Whiley DM, LeCornec GM, Mackay IM, Siebert DJ, Sloots TP.** A real-time PCR assay for the detection of *Neisseria gonorrhoeae* by LightCycler. *Diagn Microbiol Infect Dis.* 2002 Feb;42(2):85-9.
- Tabrizi SN, Chen S, Cohenford MA, Lentricchia BB, Coffman E, Shultz T,**
- 10 **Tapsall JW, Garland SM.** Evaluation of real time polymerase chain reaction assays for confirmation of *Neisseria gonorrhoeae* in clinical samples tested positive in the Roche Cobas Amplicor assay. *Sex Transm Infect.* 2004 Feb;80(1):68-71.
- van der Ende A, Hopman CT, Dankert J.** Deletion of *porA* by recombination
- 15 **between clusters of repetitive extragenic palindromic sequences in *Neisseria meningitidis*.** *Infect Immun* 1999 Jun; 67 (6):2928-34.
- Newcombe J, Cartwright K, Dyer S, McFadden J.** Naturally occurring insertional inactivation of the *porA* gene of *Neisseria meningitidis* by integration of IS1301.
- 20 **Mol Microbiol.** 1998 Oct;30(2):453-4.

Jelfs J, Munro R, Wedege E, Caugant DA. Sequence variation in the *porA* gene of a clone of *Neisseria meningitidis* during epidemic spread. *Clin Diagn Lab Immunol* 2000 May; 7 (3):390-5.

- 5 **Ho BS, Feng WG, Wong BK, Egglestone SI.** (1992) Polymerase chain reaction for the detection of *Neisseria gonorrhoeae* in clinical samples. *J Clin Pathol* 45:439-442

Table 1: NGpapLC primers and probes targeting the *N. gonorrhoeae* porA pseudogene

Designation	Sequence (5' to 3')	Position
papF:	CGGTTTCCGTGCGTTACGA	681 – 699 ^a
papR:	CTGGTTTCATCTGATTACTTTCCA	812 – 789 ^a
papP1:	CATTCAATTTGTTCCGAGTCAAAACAGC –fluorescein 730 - 757 ^a	
papP2:	LCred640 – AGTCCGCCTATACGCCTGCTACTTTTAC – Phosphate	759 - 786 ^a

^aGenbank accession number AJ223448

Table 2: An improved confirmatory *Neisseria gonorrhoeae* real-time PCR assay targeting the porA pseudogene.

N = 282	Cobas Amplicor & cppB-LC testing algorithm positive	Cobas Amplicor & cppB-LC testing algorithm negative
NGpapLC positive	79	1
NGpapLC negative	1	201*

*81 of these specimens were positive by the Cobas Amplicor assay but negative by the cppB-LC assay.

Table 3: All specimen types: PCR v Culture

N = 557	Culture positive	Culture negative
NGpapLC positive	15	2*
NGpapLC negative	0	540

* Currently, there is no assay available that could reliably be used for discrepant analysis on these specimens. However, these specimens were also positive by the cppB-LC (which can also cross-react with other *Neisseria* species). Further, these specimens provided comparable Ct values in both the NGpapLC and cppB-LC assays. This suggests that the bacterial DNA detected by these assays were at approximately the same concentration. Overall, these results suggest that these are true *N. gonorrhoeae* positive specimens.

Table 4: Cervical swabs: PCR v Culture

N = 216	Culture positive	Culture negative
NGpapLC positive	1	0
NGpapLC negative	0	215

Table 5: Urethral swabs: PCR v Culture

N = 184	Culture positive	Culture negative
NGpapLC positive	9	0
NGpapLC negative	0	175

Table 6: Throat swabs: PCR v Culture

N = 133	Culture positive	Culture negative
NGpapLC positive	1	1*
NGpapLC negative	0	131

Table 7: Anal swabs: PCR v Culture

N = 24	Culture positive	Culture negative
NGpapLC positive	4	1*
NGpapLC negative	0	19

DATED this eighth day of April 2004

THE STATE OF QUEENSLAND ACTING THROUGH ITS
DEPARTMENT OF HEALTH

by its Patent Attorneys

FISHER ADAMS KELLY

NGpapLC oligonucleotides:

NG-pap-1: CGGTTTCCGTGCGTTACGA cggtttccgttcggtacga
 NG-pap-2: CTGGTTTCATCTGATTACTTTCCA cggtttccgttcggtacga (AS))
 NG-pap-p1: CATTCAATTTGTTCCGAGTCAAAACAGC cattcaattgttccggtcaaaaacagc
 NG-pap-p2: AGTCCGCCTATACGCCTGCTACTTTTAC agtcgcctatacgccgctacttttca

Additional oligos.

NG-pap-3: TGCTACTTTCACGCTGGAAAGTA tgctactttcacgctggaaagta
 NG-pap-4: GAAAGTAATCAGATGAAACCAGTTC gaaagtaatcagatgaaaccagt
 NG-pap-5: AGGGGCATGATGCTTTCTTTTGTTC aggggcatagatgctttctttgttc
 NG-pap-6: CTGCTCGGCAGAGCGAGTGATA ctgctcggcagagcgagtga
 NG-pap-7: GCCGAGCAAGAACAAAAGAAAGCA gccgagcaagaacaaaagaaagca (AS))

Aaggtctgtattttaaatcatgttgcggaagcaacattttcaaaaaagttaatttattgttttatatt
 gaaatattatttttcaaaaataaaaatcccaaaatttaccggaattttgttccgaaaaatggttttttt
 cggggggggttaattggagactgattgggtgtttgccgatgttttagcaaatttacaaaaggaagcgc
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 tgtacggcgaaagtcaaaagctgggtgtggaaggcaggaacatccggctgcagttgaccgagccaccctcag
 aaggtcaaacgggcaatacagttactaaggccaaaagccgcacaggaacgaaagtcagtgatttcggct
 cgtttatcggttttaagggggtgggattttggcgcgcggtggaaggctgtttggcagctcgagcaaga
 cgtatccgttgcggcgcgcgacccggttgggttaacaggaatcctttatcggttggcagggcga
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 ggacagcaataataatgtggcttcgcaattgggtattttcaaacgccacgacggtatgc

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 actgcttctaccgcttcggtatacagtcgccgcgcatcagctatgcccatggtttcgacttgcgaa
 cgcagtcagaaacgcgaacataccagctatgatcaaatcatcgccggtgtcgattacgattttccaag
 cgcacttccgccatcatgtctgcggttgggtgaaacgaaataccggcatcggaactacactcaaatt
 aatgccgcctccgttggctctgcgccacaaattc

FIG.1